CCN3 increases BMP-4 expression and bone mineralization in osteoblasts

Tzu-Wei Tan[1,2], Yuan-Lin Huang[3], Jung-Tzu Chang[2], Jen-Jyh Lin[4], Yi-Chin Fong[4,5], Chien-Chung Kuo[5], Chun-Hao Tsai[5], Yen-Jen Chen[5], Horng-Chaung Hsu[5], Der-Yang Cho[6], Yi-Hung Chen[7*] and Chih-Hsin Tang[1,2*]

[1]Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung Taiwan

[2]Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan

[3]Department of Biotechnology, College of Health Science, Asia University, Taichung, Taiwan

[4]School of Chinese Medicine, China Medical University and Hospital, Taichung, Taiwan

[5]Department of Orthopaedics, China Medical University Hospital, Taichung, Taiwan

[6]Department of Neurosurgery, China Medical University Hospital, Taichung, Taiwan

[7]Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan

*: **Author for Correspondences:**

Chih-Hsin Tang,

Department of Pharmacology, School of Medicine, China Medical University

No. 91, Hsueh-Shih Road, Taichung, Taiwan

Tel: 886-4-22052121-7726; Fax: 886-4-22053764; E-mail: chtang@mail.cmu.edu.tw

Or

Yi-Hung Chen; Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan; E-mail: yihungchen@mail.cmu.edu.tw

T.W. Tan and Y.L. Huang contributed equally to this work.

ABSTRACT

The nephroblastoma overexpressed (NOV) gene, also called CCN3, regulates differentiation of skeletal mesenchymal cells. Bone morphogenetic proteins (BMPs) play important roles in osteoblast differentiation and bone formation, but the effects of CCN3 on BMP expression and bone formation in cultured osteoblasts are largely unknown. Here we found that CCN3 increased BMP-4 expression and bone nodule formation in cultured osteoblast. Monoclonal antibodies for α5β1 and αvβ5 integrins, and inhibitors of integrin-linked kinase (ILK), p38, and JNK, all inhibited CCN3-induced bone nodule formation and BMP-4 up-regulation of osteoblasts. CCN3 stimulation increased the kinase activity of ILK and phosphorylation of p38 and JNK. Inhibitors of activator protein-1 (AP-1) also suppressed bone nodule formation and BMP-4 expression enhanced by CCN3. Moreover, CCN3-induced c-Jun translocation into the nucleus, and the binding of c-Jun to the AP-1 element on the BMP-4 promoter were both inhibited by specific inhibitors of the ILK, p38, and JNK cascades. Taken together, our results provide evidence that CCN3 enhances BMP-4 expression and bone nodule formation in osteoblasts, and that the integrin receptor, ILK, p38, JNK and AP-1 signaling pathways may be involved.

Key Word: CCN3; Osteoblasts; BMP-4; ILK; p38; JNK

Running title: CCN3 increases BMP-4 expression

INTRODUCTION

Bone is a complex tissue composed of several cell types that undergo a continuing process of renewal and repair termed "bone remodeling". The two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone. When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results (Goltzman, 2002). Current drugs used to treat osteoporosis include bisphosphonates, calcitonin, and estrogen. These drugs are all bone resorption inhibitors, which maintain bone mass by inhibiting osteoclast function (Rodan and Martin, 2000). The effect of these drugs in increasing or recovering bone mass is relatively small, certainly no more than 2% per year (Rodan and Martin, 2000). It is therefore desirable, to have satisfactory bone-building agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture that is characteristic of established osteoporosis (Berg et al., 2003; Ducy et al., 2000). Because new bone formation is primarily a function of the osteoblast, agents that either increase the proliferation of the osteoblast lineage or induce differentiation of osteoblasts can enhance bone formation (Ducy et al., 2000; Lane and Kelman, 2003).

Bone morphogenetic proteins (BMPs), which constitute a family of more than 20 members, belong to the transforming growth factor-β (TGF-β) superfamily and were originally identified by their unique ability to induce ectopic cartilage and bone formation in vivo (Su et al., 2007; Wozney et al., 1988; Zhao et al., 2002). It has been shown that BMP-2, BMP-4, and BMP-7 are synthesized by osteoblasts (Canalis, 2009). BMPs play important roles in bone formation and bone cell differentiation by stimulating alkaline phosphatase activity and synthesis of proteoglycan, collagen, osteocalcin, and Rux2 (also known as CBFA1) (Axelrad and Einhorn, 2009; Chen et al., 2004). BMP-4 is a member of the BMP-2 subfamily and induces apoptosis in different cells (Trousse et al., 2001). Mechanical stress induces osteoblast differentiation, which then leads to osteogenesis

(Ikegame et al., 2001). This osteoblast differentiation seems to be accompanied by an increase in BMP-4 gene expression. Although BMP-4 expression is up-regulated under tensile stress and during distraction osteogenesis (Ikegame et al., 2001; Sato et al., 1999), the exact role of BMP-4 in these systems remains unclear.

Several lines of evidence have demonstrated that the CCN family of proteins [cysteine-rich 61(CYR61)/CCN1, connective tissue growth factor (CTGF)/CCN2, nephroblastoma overexpressed gene (NOV)/CCN3, Wnt-induced secreted protein 1 (WISP1)/CCN4, WISP2/CCN5, and WISP3/CCN6] regulates differentiation of skeletal mesenchymal cells such as muscle cells, chondrocytes, and osteoblasts (Calhabeu et al., 2006; Nishida et al., 2000; Takigawa et al., 2003). CTGF reportedly binds BMP-4 and antagonizes its activity by preventing it from binding to BMP receptors (Nishida et al., 2000). CTGF also modulates the action of BMP-9 during osteoblast differentiation (Luo et al., 2004). CCN-family genes are expressed in osteoblasts isolated from the calvariae of newborn mice, and their expression is regulated by TGF-β and BMP-2 (Parisi et al., 2006). A previous study showed that CCN affects cell migration by binding to cell surface integrin receptors (Leask and Abraham, 2006). Integrin-linked kinase (ILK), a candidate signaling molecule, has been shown to regulate integrin-mediated signaling (Hannigan et al., 1996). ILK can interact with the cytoplasmic domain of β-integrin subunits, is activated by integrin activation as well as growth factors, and is an upstream regulator of p38, JNK, and activator protine-1 (AP-1) (Sawai et al., 2006).

CCN1 and CCN2 have been reported to increase BMP expression and bone formation in osteoblasts (Luo et al., 2004; Su et al.). In humans, BMPs were recently recognized as an osteoporosis-associated gene through human polymorphism studies. However, the role of CCN3 in osteoblast differentiation and BMP expression has not been elucidated. We hypothesized that CCN3 controls osteoblast function by regulating BMP gene expression in osteoblasts. Here we show that CCN3 increases BMP-4 expression and bone mineralization in osteoblasts. In addition, integrin receptor, ILK,

p38, JNK, and AP-1 signaling pathways may be involved in increasing BMP-4 expression and bone mineralization via CCN3.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies specific for ILK, β-actin, p-p38, p38, p-JNK, JNK, p-c-Jun and c-Jun, as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies specific for glycogen synthase kinase 3β (GSK3β) and phospho-GSK3β were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody was purchased from Leinco Technology Inc. (St Louis, MO). Recombinant human CCN3 was purchased from PeproTech (Rocky Hill, NJ). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibodies specific for α5β1 (mAb2514, Clone BMB5) and αvβ5 (MAB1961Z) integrin were purchased from Chemicon. Hamster monoclonal antibodies specific for αvβ3 integrin (Clone H9.2B) was purchased from BD Biosciences. KP-392 was purchased from Kinetek Pharmaceuticals (Vancouver, Canada). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). Mouse monoclonal antibody specific for BMP-4 was purchased from R&D Systems (Minneapolis, MN, USA). The p38 dominant negative mutant was provided by Dr. J. Han (University of Texas South-western Medical Center, Dallas, TX). The JNK dominant negative mutant was provided by Dr. M. Karin (University of California, San Diego, CA, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

The murine osteoblast cell line MC3T3-E1 was purchased from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in 95% air, 5% CO_2 with α-MEM that was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Measurement of mineralized nodule formation

The level of mineralized nodule formation was evaluated as described (Tang et al., 2007). Osteoblasts were cultured in α-MEM containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks, and the medium was changed every 3 days. After incubation with CCN3 for 12 days, cells were washed twice with 20 mM Tris-buffered saline containing 0.15 M NaCl (pH 7.4), fixed in ice cold 75% (v/v) ethanol for 30 min, and air-dried. Calcium deposition was determined using alizarin red-S staining. Briefly, ethanol-fixed cells and matrix were stained for 1 h with 40 mM alizarin red-S (pH 4.2) and rinsed extensively with water. Images of the mineralized bone nodules were acquired using an Olympus microscope IX70. After photography, the bound stain was eluted with 10% (w/v) cetylpyridinium chloride, and alizarin red-S in the samples was quantified by measuring absorbance at 550 nm using a standard curve. One mole of alizarin red-S selectively binds about two moles of calcium.

Migration assay

The migration assay was performed by using Transwell (Costar, NY, USA; pore size, 8-μm) in 24-well dishes. Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing CCN3 was placed in the lower chamber. The plates were incubated for 72 h at 37℃ in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times (Hou et al., 2009; Lu et al., 2010a).

Cell proliferation

Cell proliferation was determined by the

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. After treatment with CCN3 for 72 h, cultures were washed with PBS. MTT (0.5 mg/ml) was then added to each well, and the mixture was incubated for 2 h at 37℃. Culture medium was then replaced with an equal volume of dimethyl sulfoxide to dissolve formazan crystals. After shaking at room temperature for 10 min, absorbance was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT) (Lu et al., 2010b).

Quantitative real time PCR

Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Reverse transcription was performed using 2 μg total RNA and an oligo(dT) primer. Quantitative real-time PCR (qPCR) was carried out using TaqMan® one-step PCR Master Mix (Applied Biosystems, CA). 100 ng of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. All target gene primers and probes were purchased commercially, including β-actin as an internal control (Applied Biosystems, CA). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T). BMP-4 mRNA levels were normalized to the β-actin mRNA levels and expressed relative to control using the ΔΔCt method.

Western blot analysis

Cell lysates were prepared as described (Tan et al., 2009b). Proteins were resolved by SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes. The blots were blocked with 4% bovine serum albumin for 1 h at room temperature and then probed with rabbit anti-human antibodies against p-JNK, p-p38 or BMP-4 (1:1000) for 1

h at room temperature. After three washes, the blots were incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using X-OMAT LS film (Eastman Kodak, Rochester, NY). The activities of p38 were determined using kit from Cell Signaling Technology according to the manufacturer's instructions.

ILK kinase assay

ILK enzymatic activity was assayed in osteoblasts lysed in NP-40 buffer (0.5% sodiumdeoxycholate, 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl) as reported (Tan et al., 2004; Tseng et al.). Briefly, ILK was immunoprecipitated from 250 μg lysate using anti-ILK overnight at 4℃. After immunoprecipitation, beads were resuspended in 30 μl kinase buffer containing 1 μg recombinant substrate (GSK3β fusion protein) and 200 μM cold ATP, and the reaction was carried out for 30 min at 30 ℃. The phosphorylated substrate was visualized by western blotting with anti-phospho-GSK3β. Total GSK3β was detected with the appropriate antibody.

siRNA transfection

The siRNA against human ILK, c-Jun and control (for experiments using targeted siRNA transfection; each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Synthesis of NF-κB and AP-1 decoy oligonucleotide (ODN)

We used a phosphorothioate double-stranded decoy ODN carrying the AP-1 decoy ODN sequence was 5'-TGTCTGACTCATGTC-3'/3'-ACAGACTGAGTACAG-5'. The mutated (scrambled) form

5’-TTGCCGTACCTGACTTAGCC-3’/3’-AACGGCATGGACTGAATCGG-5’ was used as a control. ODN (5 μM) was mixed with Lipofectamine 2000 (10 μg/ml) for 25 min at room temperature, and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments.

Immunofluorocytochemistry

Cells were cultured on 12-mm coverslips. After treatment with CCN3, cells were fixed with 4% paraformaldehyde at room temperature for 30 min. PBS containing 4% nonfat milk and 0.5% Triton X-100 was added to the cells, and cells were incubated with rabbit anti-c-Jun (1:100) and FITC-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St Louis, MO, USA) for 1 h, each. FITC was detected using a Zeiss fluorescence microscope.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as described (Chiu et al., 2009). DNA immunoprecipitated with anti-c-Jun was purified, extracted with phenol/chloroform, and PCR amplified across the BMP-4 promoter region [–505 to –305; (Feng et al., 1995) using primers 5'-CTGCTCACAGCCTGTTTCAA-3' and 5'-TGGGCTTCCCTGAGTTTAGA-3'.] PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by UV light.

Statistical analysis

Statistical analysis was performed using Prism 4.01software. (GraphPad Software Inc., San Diego, CA, USA). The values given are mean ± S.E.M. Statistical analysis between two samples was performed using the Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance with Bonferroni's *post hoc* test. In all cases, $p<0.05$ was considered significant.

RESULTS

CCN3 increases bone nodule formation in osteoblasts

In this study, we investigated the role of CCN3 in osteoblast proliferation and differentiation. Using an MTT assay, we found that treatment with CCN3 for 72 h did not affect the proliferation of mouse osteoblast MC3T3-E1 cells (Fig. 1A). Because osteoblast differentiation is a complicated process that includes cell proliferation and migration, we also tested the migratory ability of MC3T3-E1 cells after treatment with CCN3. Using a Transwell assay, CCN3 did not affect osteoblast migration (Fig. 1B). The formation of mineralized nodules is one of the markers of osteoblast maturation. Alizarin red staining showed that mineralized nodules formed when osteoblasts were cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. However, treatment with CCN3 for 2 weeks increased the formation of bone nodules (Fig. 1C). Therefore, CCN3 induced bone nodule formation but not proliferation or migration in cultured mouse osteoblasts.

CCN3 increases bone mineralization through BMP-4 up-regulation in osteoblasts

Given the crucial role of BMPs in osteoblast differentiation, we tested whether CCN3 mediates this alteration in osteoblast differentiation through regulation of BMP expression. BMP-2, -4, and -7 may be the most potent molecules that induce osteoblast lineage–specific differentiation (Axelrad and Einhorn, 2009). Therefore, we explored the expression of these possible target genes during CCN3-induced osteoblast differentiation. Treatment of cells with CCN3 increased mRNA expression of BMP-4 in a concentration-dependent manner (Fig. 2A). CCN3 also increased BMP-4 protein expression as determined by western blotting (Fig. 2B). In contrast, stimulation of osteoblasts with CCN3 (30 ng/ml) did not increase BMP-2 mRNA expression (Fig. 2C), and only slightly increased BMP-7 mRNA expression (Fig. 2C). To determine whether induction of BMP-4 is critical for CCN3-mediated osteoblast differentiation, we assessed

the inhibitory effect of a neutralizing antibody against BMP-4. Our data showed that CCN3-induced bone nodule formation was significantly decreased after treatment with the neutralizing antibody (Fig. 2D). These data showed that CCN3 induced differentiation of osteoblasts via up-regulation of BMP-4.

Integrin receptor is involved in CCN3-mediated BMP-4 expression in osteoblasts

To determine whether the CCN3-induced increase in BMP-4 expression required transcription or translation, osteoblasts were stimulated with CCN3 in the absence or presence of the transcription inhibitor actinomycin D or the translation level inhibitor cycloheximide, and BMP-4 expression was determined. As shown in Fig. 3A-C, the CCN3-mediated induction of BMP-4 expression was abolished by either actinomycin D or cycloheximide. Taken together, these findings demonstrated that the induction of BMP-4 by CCN3 in osteoblasts depended on *de novo* protein synthesis. Here, we found that cycloheximide (translation level inhibitor) also reduced BMP-4 mRNA expression. Therefore, the reduction of mRNA was due to decreased of protein expression. A previous study showed that CCNs affect cell function by binding to cell-surface integrin receptors (Tan et al., 2009a). We therefore hypothesized that the integrin-signaling pathway may be involved in CCN3-mediated BMP-4 expression and bone nodule formation. Pretreatment of cells with anti-α5β1 or anti-αvβ5 mAb, but not anti-αvβ3 mAb, markedly inhibited CCN3-induced BMP-4 expression and bone nodule formation (Fig. 3D-F).

The signaling pathways of ILK, p38 and JNK are involved in the potentiating action of CCN3

ILK, a 59-kDa serine/threonine protein kinase, is regulated in integrin signaling (Tan et al., 2004). To determine whether ILK is involved in CCN3-induced BMP-4 expression, the ILK inhibitor KP-392 and ILK-specific siRNA were used to inhibit ILK activity.

Transfection of ostoeblasts with ILK siRNA reduced ILK protein expression as seen by western blotting (Fig. 4A), and it antagonized the potentiating effect of CCN3 on BMP-4 expression (Fig. 4B). Pretreatment of osteoblasts with KP-392 inhibited CCN3-induced BMP-4 expression (Fig. 4B,C) as well as bone nodule formation (Fig. 4E). We next directly measured the kinase activity of ILK in response to CCN3 stimulation by immunoprecipitating of ILK from lysates. Immunoprecipitated proteins were analyzed by western blotting for phosphorylation of GSKβ on Ser^{9}. Fig. 4D shows that CCN3 stimulation of osteoblasts increased ILK activity in a time-dependent manner. It has been reported that ILK is an upstream regulator of p38 and JNK (Zhang et al., 2006). Therefore, we also examined whether CCN3 stimulation enhanced p38 and JNK activation. Pretreatment of cells for 30 min with the p38 inhibitor SB203580 or the JNK inhibitor SP600125 inhibited CCN3-induced BMP-4 expression and bone nodule formation (Fig. 5A-D). Transfection of cells with dominant-negative mutants of p38 and JNK also reduced CCN3-mediated BMP-4 up-regulation (Fig. 5A&B). Furthermore, CCN3 induced p38 and JNK phosphorylation (Fig. 5E), but these activities were markedly decreased if osteoblasts were pretreated for 30 min with KP-392 (Fig. 5F). Taken together, these results indicated that the ILK, p38, and JNK pathways are involved in CCN3-induced BMP-4 expression and bone nodule formation.

AP-1 signaling pathway is involved in CCN3-mediated BMP-4 expression

As previously mentioned, AP-1 activation is necessary for BMP-4 production (Xu et al., 1996). To examine whether AP-1 activation is involved in CCN3-induced BMP-4 expression, the AP-1 inhibitors curcumin and tanshinone IIA were used. Fig. 6A&B&D show that pretreatment of osteobalsts with curcumin or tanshinone IIA inhibited CCN3-induced BMP-4 expression and bone nodule formation. Furthermore, transfection of cells with a specific AP-1-binding site (decoy AP-1 ODN), but not a scrambled decoy (ODN) also reduced CCN3-induced BMP-4 expression (Fig. 6C). These results indicated

that AP-1 activation is important for CCN3-induced BMP-4 expression and bone nodule formation. AP-1 activation was further evaluated by analyzing the c-Jun phosphorylation, and c-Jun translocation into the nucleus. Cells were transfected with c-Jun siRNA to suppress c-Jun expression (Fig. 7A; upper panel). CCN3-induced BMP-4 expression was inhibited by c-Jun siRNA, but not by the control siRNA (Fig. 7A). As seen by western blotting, treatment of cells with CCN3 resulted in a marked phosphorylation of c-Jun (Fig. 7B). Immunofluorescence staining showed that stimulation of cells with CCN3 increased c-Jun translocation into the nucleus, and that this effect was attenuated by KP392, SB203580, or SP600125 (Fig. 7C). It has been reported that the AP-1-binding site between nucleotides -363 and -357 is important for activation of the BMP-4 gene (Feng et al., 1995). Thus, we next investigated whether c-Jun could bind to the AP-1 element on the BMP-4 promoter after CCN3 stimulation. The recruitment of c-Jun to the ICAM-1 promoter (–505 to –305) was assessed by chromatin immunoprecipitation. The binding of c-Jun to the AP-1 element of the BMP-4 promoter was occurred after CCN3 stimulation (Fig. 7D). The c-Jun translocation into nucleus and the binding of c-Jun to the AP-1 element by CCN3 were attenuated by the KP392, SB203580 and SP600125 (Fig. 7C&D). Taken together, these data suggested that activation of ILK, p38, JNK and c-Jun are required for CCN3-induced AP-1 activation in osteoblasts

Integrin receptor, ILK, p38, JNK and AP-1 signaling pathways are involved in CCN3-mediated BMP-4 expression in primary osteoblasts and mesenchymal stem cells.

Altered mechanical signals are known to induce local production of soluble factors, including CCN family members that promote changes in the physiological properties of tissues and organs. In vivo, it is possible that a local increase in CCN3 in the bone marrow induces differentiation of osteoblast progenitors. We found that CCN3 increased BMP-4 expression in primary osteoblasts and mesenchymal stem cells (C3H10T1/2 cells; Fig. 8). In addition, integrin mAbs, KP392, SB203580, SP600125, curcumin, and

tanshinone IIA all inhibited CCN3-induced BMP-4 expression (Fig. 8). Therefore, the same signaling pathways are involved in primary osteoblasts and mesenchymal stem cells. Our data thus provide evidence that these pathways play important roles in maintaining the bone microenvironment.

DISCUSSION

NOV, also called CCN3, was identified as a gene that is expressed in avian nephroblastoma induced by myeloblastosis-associated virus (Perbal et al., 1985). Subsequently, human and rodent NOV was cloned, and its expression was identified in a variety of mesenchymal cells during development (Martinerie et al., 1992). The function of NOV may differ between specific cell types. In neural tissues, the expression pattern of NOV suggests a positive role in differentiation (Su et al., 2001), whereas in myoblasts it activates Notch signaling, resulting in inhibition of muscle development (Sakamoto et al., 2002). Recent work showed that CCN3 is a crucial component in the bone marrow microenvironment in response to mechanical stress (Katsube et al., 2009). Other studies showed that CCN3 expression is very low in normal proliferating osteoblasts and mesenchymal stem cells, and that its overexpression in osteosarcomas is associated with poor patient prognosis (Katsuki et al., 2008; Perbal et al., 2008). Our data demonstrated that CCN3 increased BMP-4 expression and bone nodule formation in osteoblast. In a physiological context, the source for secreted CCN3 (whether autocrine, paracrine, or even systemic) needs further examination. In this study, we found that CCN3 mostly effectively induced BMP-4 expression at 30 ng/ml. However, physiological CCN3 conditions are complex and we cannot, at this time, compare these in vitro results to physiological conditions.

BMPs play an important role in bone formation and remodeling (Sykaras and Opperman, 2003). Here we found that CCN3 increased BMP-4 expression and slightly increased BMP-7 expression. However, treatment of cells with anti-BMP-7 also slightly reduced the CCN3-induced mineralization (data not shown). Therefore, a role for BMP-7 in CCN3-mediated effects cannot be ruled out. In addition, we cannot rule out the possibility that the BMP-4-specific antibody did not also target BMP-2 and BMP-7. During osteoblast differentiation, BMP-4 mRNA is induced and maintains the sustained phenotype of mature osteoblasts (Anderson et al., 2000). Previous studies have indicated

that BMP-4 gene regulation during limb morphogenesis and osteoblast differentiation may involve multiple mechanisms and signaling pathways, including prostaglandin E2, retinoic acid, Hoxa13, Gli2/3, interferon, and interleukins (Garrett et al., 2003; Zhao et al., 2006). There are binding sites for a number of transcription factors, including AP-1, in the promoter region of the BMP-4 gene (Feng et al., 1995). The results of our present study show that AP-1 activation contributes to CCN3-induced BMP-4 expression in osteoblasts, and that inhibitors of the AP-1-dependent signaling pathway, including curcumin and tanshinone IIA, down-regulate CCN3-induced BMP-4 expression and bone nodule formation. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of our study show that CCN3 induced c-Jun phosphorylation, increased c-Jun translocation into the nucleus, and induced binding of c-Jun to the AP-1 element in the BMP-4 promoter. The c-Jun translocation and binding to the AP-1 element were attenuated by KP392, SB203580 and SP600125. Furthermore, c-Jun siRNA abolished CCN3-induced BMP-4 expression in osteoblasts. These results indicate that CCN3 might act through the ILK, p38, JNK, c-Jun, and AP-1 pathways to induce BMP-4 expression in osteoblasts. Moreover, we found that the same signaling pathways are involved in primary osteoblasts and mesenchymal stem cells. Our data provide evidence that these pathways play important roles in the bone microenvironment.

Integrins link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth and differentiation (Danen, 2009). A previous study showed that CCN3 affects cell functions such as migration through binding to cell surface integrin receptors (Leask and Abraham, 2006). Here we used integrin-specific antibodies to determine the role of integrins, and found that anti-α5β1 and anti-αvβ5 mAbs inhibited CCN3-induced BMP-4 expression,

indicating the possible involvement of α5β1 and αvβ5 integrin activation in CCN3-induced BMP-4 expression in osteoblasts. In addition, α5β1 and αvβ5 mAbs also reduced CCN3-enhanced bone nodule formation. These two integrins reportedly play an important role in osteogenesis (Siebers et al., 2005). These data also suggest that CCN3-induced BMP-4 expression may occur via activation of the α5β1 and αvβ5 integrin.

ILK, a potential candidate-signaling molecule, has been shown to regulate integrin-mediated signaling (Lee et al., 2006; Tan et al., 2004). Our current study showed that CCN3 stimulation increased the kinase activity of ILK. Treatment with the ILK inhibitor KP-392 attenuated CCN3-induced BMP-4 expression and bone nodule formation. Furthermore, the ILK siRNA antagonized the CCN3-induced potentiation of BMP-4 expression. Therefore, ILK activation is involved in CCN3-induced BMP-4 expression and bone formation in the cultured osteoblasts. Focal adhesion kinase (FAK) has also been shown to be capable of regulating integrin-mediated signaling. However, transfection of cells with FAK siRNA did not reduce CCN3-induced BMP-4 expression (data not shown). Therefore, FAK is not involved in CCN3-mediated BMP-4 expression, and the role of ILK is specific. Whether other signaling molecules link integrin to BMP-4 expression are connected to integrin and BMP-4 needs further examination. ILK may regulate cell functions by promoting p38 and JNK activation (Zhang et al., 2006). p38 and JNK participated in bone development and homeostasis (Schindeler and Little, 2006), and our results demonstrate that pretreatment of osteoblasts with p38 or JNK inhibitors antagonized the increase of BMP-4 expression and bone nodule formation by CCN3 stimulation. In this study, we found both p38 and JNK inhibitor reduced CCN3-mediated bone mineralization. Therefore, p38 and JNK are simultaneously involved bone formation and mineralization. Moreover, we found that CCN3 enhanced p38 and JNK phosphorylation in osteoblasts and that. Pretreatment of cells with KP-392 reduced CCN3-induced p38 and JNK phosphorylation. These effects implicate ILK-dependent

p38 and JNK activation in CCN3-induced BMP-4 expression and bone formation.

In conclusion, the signaling pathway involved in CCN3-induced BMP-4 expression and bone nodule formation in osteoblasts has been explored. CCN3 increased BMP-4 expression and bone nodule formation by activating of $\alpha5\beta1/\alpha v\beta5$ integrins, ILK, p38, and JNK, which enhanced binding of c-Jun to the AP-1 site, resulting in transactivation of BMP-4 production.

ACKNOWLEDGMENTS

This work was supported by grants from National Science Council of Taiwan (NSC99-2320-B-039-003-MY3) and China Medical University (CMU99-ASIA-10). We thank Dr. J. Han for providing p38 mutant; Dr. M. Karin for providing JNK dominant negative mutant.

FIGURE LEGENDS

Fig. 1 CCN3 increases bone mineralization in cultured osteoblasts.

(A) Cells were incubated with CCN3 for 72 hr, and cell viability was measured by MTT assay (n=5). (B) Cells were incubated with CCN3, and *in vitro* migration was measured with the Transwell after 72 hr (n=5). (C) Osteoblasts were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. The cells were concomitantly treated with CCN3. At the end of experiment, cultures were fixed in 75% ethanol, and mineralized nodule formation was assessed by alizarin red-S staining (upper panel). The bound staining was eluted with a solution of 10% cetylpyridinium chloride and quantified using a microtiter plate reader (lower panel) (n=5). Results are expressed as the mean ± S.E.

Fig. 2 CCN3 increases BMP-4 expression in cultured osteoblasts.

(A) Cells were incubated with CCN3 (3-100 ng/ml) for 24 hr, and BMP-4 mRNA expression was measured by qPCR (n=5). (B) Cells were incubated with CCN3 (30 ng/ml) for 24 hr, and BMP-4 protein expression was measured by Western blot (n=5). (C) Cells were incubated with CCN3 (30 ng/ml) for 24 hr, and BMP-2, -4 and -7 mRNA expression was measured by qPCR (n=5). (D) Osteoblasts were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. The cells were concomitantly treated with CCN3 (30 ng/ml) plus BMP-4 neutralizing antibody (mAb). The mineralized nodule formation was assessed by alizarin red-S staining (n=5). Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

Fig. 3 Involvement of integrin receptor in CCN3-induced BMP-4 expression in

osteoblasts.

(A-C) Cells were pretreated with actinomycin D (1 μM) or cycloheximide (1 μM) for 30 min followed by stimulation with CCN3 (30 ng/ml), and BMP-4 mRNA and protein expression was measured by qPCR and Western blot. (n=5). (D&E) Cells were pretreated with α5β1, αvβ5 and αvβ3 mAb (10 μg/ml) for 30 min followed by stimulation with CCN3 (30 ng/ml), and BMP-4 mRNA and protein expression was measured by qPCR and Western blot (n=5). (F) Cells were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. The cells were concomitantly treated with CCN3 (30 ng/ml) plus α5β1 or αvβ5 neutralizing antibodies (10 μg/ml). The mineralized nodule formation was assessed by alizarin red-S staining (n=5). Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

Fig. 4 ILK is involved in CCN3-mediated BMP-4 expression in osteoblasts.

(A) Cells were transfected with ILK siRNA for 24 hr, and ILK expression was examined by Western blot. (B&C) Cells were pretreated with ILK inhibitor of KP-392 (10 μM) for 30 min or transfected with ILK siRNA for 24 hr followed by stimulation with CCN3 (30 ng/ml) for 24 hr, and BMP-4 mRNA and protein expression was measured by qPCR and Western blot (n=5). (D) Cells were treated with CCN3 (30 ng/ml) for indicated time intervals, and cell lysates were immunoprecipitated (*IP*) with an antibody specific for ILK. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-pGSK3β or GSK3β (n=5). (E) Cells were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for

2 weeks. The cells were concomitantly treated with CCN3 (30 ng/ml) plus KP-392 (10 μM). The mineralized nodule formation was assessed by alizarin red-S staining (n=5). Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

Fig. 5 p38 and JNK are involved in CCN3-mediated BMP-4 expression in osteoblasts.

(A-C) Cells were pretreated with p38 inhibitor SB203580 (10 μM) and JNK inhibitor SP600125 (10 μM) for 30 min or transfected with p38 and JNK mutant for 24 h followed by stimulation with CCN3 (30 ng/ml) for 24 hr, and BMP-4 mRNA and protein expression was measured by qPCR and Western blot (n=5). (D) Cells were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. The cells were concomitantly treated with CCN3 (30 ng/ml) plus SB203580 (10 μM) or SP600125 (10 μM). The mineralized nodule formation was assessed by alizarin red-S staining (n=5). (E) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals, and p-38 and p-JNK expression were determined by Western blot (n=5). (F) Cells were pretreated of KP392 for 30 min followed by stimulation with CCN3 (30 ng/ml) for 60 min, and p-38 and p-JNK expression were measured by Western blot (n=5). Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

Fig. 6 CCN3 induces BMP-4 up-regulation through AP-1.

(A&B) Cells were pretreated with curcumin (10 μM) and tanshinone IIA (10 μM) followed by stimulation with CCN3 (30 ng/ml) for 24 hr, and BMP-4 mRNA and protein expression was measured by qPCR and Western blot (n=5).

(C) Cells were transfected with AP-1 ODN or scramble ODN for 24 h followed by stimulation with CCN3 (30 ng/ml), and BMP-4 mRNA expression was measured by qPCR (n=5). (D) Cells were plated in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks. The cells were concomitantly treated with CCN3 (30 ng/ml) plus curcumin (10 μM) or tanshinone IIA (10 μM). The mineralized nodule formation was assessed by alizarin red-S staining. (n=5) Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group

Fig. 7 ILK, p38 and JNK pathways are mediated CCN3-induced AP-1 activation.

(A) Cells were transfected with c-Jun siRNA for 24 h followed by stimulation with CCN3 (30 ng/ml) for 24 hr, and BMP-4 mRNA expression was measured by qPCR. (n=5) (B) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals, and p-c-Jun expression was determined by Western blot. (C&D) Cells were pretreated with KP392, SB203580 and SP600125 for 30 min. Then they were followed by stimulation with CCN3 for 120 min, and c-Jun immunofluorescence staining and chromatin immunoprecipitation assay was examined (n=5). Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

Fig. 8 Integrin, ILK, p38, JNK, and AP-1 pathways are involved in CCN3-increased BMP-4 expression in primary osteoblasts and mesenchymal stem cells.

Primary osteoblasts or C3H10T1/2 cells were treated with α5β1 mAb, αvβ5 mAb, KP392, SB203580, SP600125, curcumin or tanshinone IIA for 30 min followed by stimulation with CCN3 for 24 hr, and BMP-4 mRNA expression

was measured by qPCR. Results are expressed as the mean ± S.E. *: $p<0.05$ as compared with control group. #, $p < 0.05$ compared with CCN3-treated group.

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